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(71) Applicant
Biomatrix Inc. (USA-Delaware),
65 Railroad Avenue, Ridgefield, New Jersey 07657, United States of America

(72) Inventors
Endre A. Balazs,
Adolf Leshchiner

(74) Agent and/or Address for Service
Lloyd Wise Tregear & Co.,
Norman House, 105-109 Strand, London WC2R 0AE

# (54) Insolubilized biocompatible hyaluronic acid preparations

(57) Water insoluble, biocompatible hyaluronic acid preparations are made by subjecting hyaluronic acid to treatment with a cross-linking agent selected from formaldehyde, dimethylolurea, dimethylolethylene urea, ethylene oxide, a polyaziridine, a polyisocyanate and divinyl sulphone. The preparations may be used in such *in vivo* applications as in artificial heart valves, vascular grafts etc.

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SPECIFICATION

**Water insoluble preparations of hyaluronic acid and processes therefor**

The present invention relates to biocompatible water insoluble preparations of hyaluronic acid ("HA") which, because of their biocompatibility enables them to be used in numerous in vivo applications, such as various prosthetic devices including artificial heart valves, vascular grafts, etc. The water insoluble (or cross-linked) HA can also be used to modify various polymer articles which can likewise be used in numerous in vivo applications. The invention also relates to processes for making these preparations.

Hyaluronic acid is a known, naturally occurring material which has many applications in medicine and biology. See, for example, E.A. Balazs U.S. Patent No. 4,272,522 and publications cited therein.

Cross-linked gels of hyaluronic acid are known, having been described by Laurent et al in Acta Chem. Scand. 18 (1964 No. 1; p. 274-5).

The present invention is directed to water insoluble preparations of hyaluronic acid (HA) which are biocompatible. As used herein, the term HA includes not only hyaluronic acid, but the acid addition salts thereof as well, such as the sodium potassium, calcium, etc. salts. Because of their biocompatibility, they can be used in numerous in vivo applications both per se, and in combination with various polymeric materials which have been modified by the inclusion therein of such water insoluble preparations.

In accordance with the present invention there is provided a method of making a water insoluble hyaluronic acid preparation, said method comprising subjecting hyaluronic acid or an acid addition salt thereof to treatment with a cross-linking agent selected from formaldehyde, dimethylol urea, dimethylolethylene urea, ethylene oxide, a polyaziridine, a polyisocyanate and divinyl sulfone.

In specific embodiments, the invention is directed to water insoluble preparations of hyaluronic acid including the following types of materials:

1. cross-linked hyaluronic acid powder;
2. cross-linked hyaluronic acid film;
3. cross-linked gel of hyaluronic acid;
4. cross-linked hyaluronic acid film reinforced with a polyethylene terephtalate knitted fabric, as well as other knitted fabrics; and
5. particulate materials coated with cross-linked hyaluronic acid.

The cross-linking agents that can be used to make the instant preparations include:

1. formaldehyde;
2. dimethylol urea;
3. dimethylolethylene urea;
4. polyaziridinyl compound;
5. ethylene oxide;
6. polyisocyanate; and
7. divinyl sulfone.

According to one specific embodiment of the invention, an insoluble hyaluronic acid gel is obtained by cross-linking hyaluronic acid with divinyl sulfone (DVS) in water solution at pH higher than 9 at room temperature, ie about 20°C. Depending upon the concentration and molecular size of the hyaluronic acid in the solution, the hyaluronic acid/divinyl sulfone ratio and reaction time, the swelling ratio of the gel can vary over broad limits, ie from 20 to 2000. The swelling ratio depends substantially on the ionic strength and the hydrogen ion concentration of the medium and decreases with the ionic strength.

Alternatively, DVS can be used as a cross-linking agent under reflux at elevated temperatures (ca. 60-65°C).

The following examples (wherein all parts given are by weight unless otherwise specified) illustrate several embodiments of the invention.

*Example 1*

To a water-acetone mixture a 37% by weight water solution of formaldehyde and concentrated hydrochloric acid were added. The mixture obtained was of the following compositions (% by wt): $CH_2O$, 0.27; HCl, 0.19; water/acetone ratio 1:28. Sodium hyaluronate powder (0.5 g) was refluxed in 50 ml of the mixture for 10 minutes. Then the powder was filtered off, washed thoroughly with a water/acetone 1:3 mixture, then with acetone, and dried in a vacuum oven. The hyaluronic acid powder obtained was insoluble in water and contained 1.41% of combined $CH_2O$.

*Example 2*

The above example was repeated with the cross-linking mixture of the following composition (% by wt): $CH_2O$, 2.5; HCl, 0.38; water/acetone ratio, 1:2. The $CH_2O$ content of the product was 5.3%.

In Examples 1 and 2, the cross-linking of a hyaluronic acid powder was performed in water-acetone mixtures. By changing the water/acetone ratio and the $CH_2O$ concentration, it is possible to control the swelling ratio of the product. Thus, the swelling ratio was 178% for the product of Example 1 and 230% for that of Example 2. The swelling ratio can be reduced by increasing the amount of acetone in the mixture and the $CH_2O$ concentration.

The following examples illustrate the use of a polyaziridine compound as the cross-linking agent. This polyaziridine type compound cross-links hyaluronic acid under dry conditions and at ambient temperature which is very important in the case of hyaluronic acid as the latter is a heat sensitive polymer.

*Example 3*

To 113.0 gm of a sodium hyaluronate solution in water (concentration 14.2 mg/ml), 0.42 g of polyaziridine compound - cross-linker CX-100 (Polyvinyl chemical) was added. The molar ratio of cross-linking agent to hyaluronic acid was 0.5. The mixture was cast in a glass plate as a 5mm thick layer and allowed to dry off at room temperature for 2 days. A clear film of cross-linked hyaluronic acid was obtained which was not soluble in water and had swelling ratio in water of 160%.

0.5 g of a dry sodium hyaluronate powder was mixed with 50 ml of a 1% solution of cross-linker CX-100 in acetone, kept for 5 minutes and filtered off. The powder dried off in air for 2 hours, then washed several times with water and dried in a vacuum oven at 40°C for 4 hours. The swelling ratio of the cross-linked powder in water was 135%.

The following example illustrates the use of the polyaziridine compound for obtaining cross-linked hyaluronic acid with a high degree of swelling.

*Example 5*

0.6 g of solid sodium hyaluronate was mixed with 9.2 g of 0.6% by weight solution of cross-linker CX-100 in water. The solution obtained had a molar ratio of CX-100 to sodium hyaluronate = 0.1. The sodium hyaluronate content in the solution was 6.04% by weight. The pH of the very viscous mixture obtained was adjusted to 2.5 with 2% HCL. The resulting film was readily soluble in water. The film was heated at 60°C for 30 minutes. The heat treatment provided a strong and water insoluble film.

*Example 6*

Fiber-like sodium hyaluronate (0.1093 g) was mixed with 25 ml of a 1% solution of polyisocyanate (Desmodur N-75, Mobay Chemical Corp.) in acetone and the mixture was refluxed for 10 minutes. The precipitate was separated and washed three times with acetone, dried in vacuum at 45 mm Hg and 60°C for 30 minutes and, finally, in a vacuum dessicator over phosphorus pentoxide. The product obtained (0.1127 g) was insoluble in water and had a degree of swelling of 120%.

The following example illustrates the use of dimethylolethylene urea for cross-linking hyaluronic acid.

*Example 7*

6.0 g of sodium hyaluronate solution in water (concentration 9.8 mg/ml) were mixed with 0.017 g of N,N'-dimethylolethylene urea and 0.005 g of zinc nitrate. The mixture was cast onto a glass plate and left to dry off overnight. The obtained film was heat treated at 110°C for 15 minutes. It became insoluble in water and had a degree of swelling of 145%.

The following examples illustrate the use of divinyl sulfone for cross-linking hyaluronic acid.

*Example 8*

35 ml of sodium hyaluronate solution containing 0.401 g (1 mole) of divinyl sulfone. The pH of the mixture was adjusted to approximately 8.5-9 with a 1% solution of sodium hydroxide. A film was obtained from the mixture by casting it onto a glass plate and drying it overnight at room temperature. This film was readily soluble in water. The film was heated at 60°C for 30 minutes. The heat treatment provided a strong and water insoluble film.

*Example 9*

A dry film of non cross-linked hyaluronic acid was put into a solution of 0.6 g of divinyl sulfone in a mixture of 26 g of acetone and 13 g of water and kept there for 10 minutes. The film was removed from the solution, dried in air for 10 minutes and then heated in an oven for 20 minutes at 65°C. A strong cross-linked film of hyaluronic acid was obtained.

*Example 10*

2.95 gm of air-dry sodium hyaluronate were mixed with 57.35 gm of an 0.2N solution of NaOH in water and stirred with a glass rod until completely dissolved. Then 1.0 gm of divinyl sulfone was stirred into the mixture and the latter was left for one hour at room temperature. The mixture turned into a hard gel. The gel was put into a Vir-Tis "45" homogenizer along with 100 ml of $H_2O$ and treated for 5 minutes at 30,000 rpm. Highly swollen small particles were obtained. The particles were washed several times with water and filtered off with suction on a glass filter. To determine the swelling ratio, about 1 gm of the gel was put in a 15 ml glass filter which, in turn, was put into a plastic centrifuge tube. The gel was centrifuged for 30 minutes at 3,000 rpm. The pressed out water collected at the bottom of the tube. The hyaluronic acid concentration in the gel was found to be 0.21%, ie the swelling ratio in water was 476.

*Example 11*

The procedure described in Example 10 was repeated but the gel obtained was dispersed in an 0.15 M solution of NaCl in water, and the particles were washed in the same solution. The hyaluronic acid concentration in the gel after centrifugation was 1.29% and the swelling ratio was 77.5.

*Example 12*

1.0 gm of air-dry sodium hyaluronate was dissolved in 9.0 gm of 0.2 M NaOH while stirring with a glass rod. 0.33 gm of divinyl sulfone was stirred into the viscous solution obtained and the mixture was left to stand for 20 hours at room temperature. The hard gel obtained was treated as described in Example 10. The hyaluronic acid concentration in the gel after centrifugation was 4.30%, ie the swelling ratio was 23.6.

The biocompatibility of the preparations according to the invention was demonstrated by the test procedure hereafter described.

*Example 13 - Blood Compatibility Test*

Release of $^3$H-serotonin by human platelets was used in preliminary studies to assess the blood reactivity of samples prepared according to Examples 3 and 10. Normal human venous blood was drawn into plastic syringes and immediately transferred to plastic tubes containing 3.8% sodium citrate (one part citrate to nine parts whole blood). Platelet rich plasma was prepared by centrifugation at 4°C for 15 minutes at 125 x g and removed by serological pipet to a plastic or siliconized test tube. $^3$H-serotonin ($^3$H-5-hydroxytryptamine), $^3$H-5HT; New England Nuclear, 26.3 Ci/mmol, Im Ci/ml ethanol-water) was added to platelet rich plasma (PRP). 0.2-0.5 ul/ml PRP, and incubated for 15 minutes at 30°C. In the assay, siliconized or polypropylene test tubes were used; thrombin was used as a positive control, coated and uncoated samples were tested.

*Example 4*

1.0-2.0 ml of $^3$H-5HT - PRP was added to each of duplicate test tubes containing samples to be assayed; a 50 ul aliquot was removed from the control mixture for determination of total radioactivity. Following the appropriate incubation period (10-120 minutes) 0.2-0.5 ml aliquots of the suspension were removed and centrifuged over silicon oil in an Eppendorf microfuge for 2 minutes at 12,000 x g. 50 ul of the supernatant was removed from each tube, mixed with 5 ml of liquid scintillation fluid, and radioactivity measured by beta-spectrometry. The amount of $^3$H-5HT released by thrombin or the test samples was the increment in radioactivity of the supernatant (radioactivity of experimental samples minus radioactivty of control). The test materials did not induce significant platelet release of $^3$H-5HT for up to 120 minutes.

CLAIMS

1. A method of making a water insoluble hyaluronic acid preparation, said method comprising subjecting hyaluronic acid or an acid addition salt thereof to treatment with a cross-linking agent selected from formaldehyde, dimethylol urea, dimethylolethylene urea, ethylene oxide, a polyaziridine, a polyisocyanante and divinyl sulfone.

2. A method according to Claim 1, wherein said hyaluronic acid, or salt thereof, is in the form of a powder, film or gel.

3. A method according to Claim 1 or Claim 2, wherein the cross-linking agent is formaldehyde and treatment is effected in an aqueous medium at reflux temperature.

4. A method according to Claim 1 or Claim 2, wherein the cross-linking agent is a polyaziridine and treatment is effected under dry conditions at ambient temperature.

5. A method according to Claim 1 or Claim 2, wherein the cross-linking agent is a polyisocyanate and treatment is effected in acetone at reflux temperature.

6. A method according to Claim 1 or Claim 2, wherein the cross-linking agent is dimethylolethylene urea and treatment is effected at about 110°C.

7. A method according to Claim 1 or Claim 2, wherein the cross-linking agent is divinyl sulfone.

8. A method according to Claim 7, wherein the treatment is effected at about 60-65°C.

9. A method according to Claim 7, wherein the treatment is effected at about 20°C in an aqueous alkaline medium.

10. A method according to Claim 9, wherein treatment is effected at a pH in excess of about pH 9.

11. A method according to Claim 9 or Claim 10, wherein treatment is effected for 1-20 hours.

12. A method according to any one of Claims 9-11, wherein the ratio of hyaluronic acid to divinyl sulfone is about 3:1 by weight.

13. A method of making a water insoluble hyaluronic acid preparation, substantially as described in any one of the Examples herein.

14. The product of a method according to any preceding claim.

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